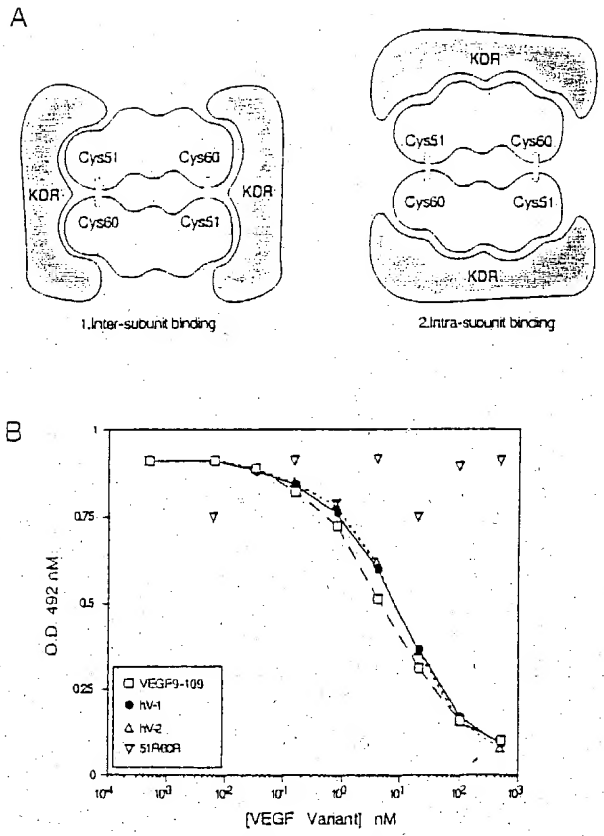

FIG. 2. *Panel A*, inter-subunit and intra-subunit models for binding two molecules of KDR receptor to dimeric VEGF. The inter-subunit disulfide bonds in VEGF are indicated. *Panel B*, displacement of biotinylated $VEGF_{1-109}$ from binding to monomeric KDR 1–7 by a monomeric form of $VEGF_{1-109}$ containing the double mutant C51R/C60R, a heterodimeric form of $VEGF_{1-109}$ that possesses either both binding sites (hV-2), or a single site at one pole of the hormone (hV-1) and the wild-type $VEGF_{1-109}$ with IC_{50} values >>500, 10, 8.9, and 4.6 nM, respectively. Dilutions of these VEGF variants were added with fixed amounts of biotinylated VEGF (1 nM) to KDR (0.5 nM) and incubated for 18 h. The complex was captured with a mAb to KDR (MAKD5) as described under "Experimental Procedures."

The IgG-like Domains 2–3 in KDR Are Sufficient for High Affinity Binding—To determine the minimal IgG-like domain requirements for binding of KDR to $VEGF_{1-109}$, a series of deletions were produced in which each of the seven IgG-like domains were deleted from the carboxyl terminus of the extracellular domain. The deletion variants were expressed initially as dimeric proteins by fusion to the CH2-CH3 domain of an antibody (KDR-IgG). This was done to facilitate purification on a Protein A affinity column (12) and to compare their affinities to monomeric forms of KDR.

The choice of deletion junction was based on homology to other members of the IgG superfamily (16, 17). Systematic carboxyl-terminal domain deletions had virtually no effect on affinity for VEGF until IgG-like domain 3 was deleted (Table I); KDR 1–2 had an affinity that was >1000-fold reduced relative to KDR 1–3 but did show specific binding at concentrations above 2 μM (data not shown). A variant of KDR missing the first NH_2-terminal domain, KDR 2–3, bound nearly as well as the full-length KDR (Table I). These data suggest that domains 2–3 are most important for high affinity binding.

To determine if these deletions had caused misfolding of the molecules, we analyzed their binding to three different anti-KDR monoclonal antibodies (Table I), one of which (MAKD6) blocks binding of VEGF. The antibodies bound to nearly all the deletion variants with affinities virtually identical to the full-length KDR 1–7 (EC_{50} ~1 nM). Deletion of domain 1 caused complete disruption for binding of the non-neutralizing antibodies (MAKD1 and MAKD5) but not the neutralizing antibody (MAKD6). Thus, the deletions do not grossly disrupt the structure of the molecules and locate the epitopes for MAKD1 and MAKD5 to domain 1 and for MAKD6 to domain 2. The fact that the antibody MAKD6, which blocks binding of VEGF, binds to domain 2 further supports the importance of domain 2 for binding VEGF.

TABLE I
Analysis of deletions of the seven IgG-like domains in the extracellular domain of KDR

	K_d (pM),[a] $VEGF_{1-109}$	EC_{50} (nM)		
		Non-blocking		Blocking
		MAKD1	MAKD5	MAKD6
KDR 1–7-IgG	51 (±11)	2.11	1.51	0.45
KDR 1–5-IgG	143 (±24)	2.13	1.05	0.41
KDR 1–4-IgG	57 (±12)	1.74	0.95	0.44
KDR 1–3-IgG	101 (±18)	1.86	1.12	0.51
KDR 1–2-IgG	>100,000	2.04	1.02	0.60
KDR 2–3-IgG	100	ND[b]	ND[b]	0.50
KDR1–7 monomer	4990 (±700)	1.89	1.59	0.64
KDR1–3 monomer	3350 (±1000)	1.81	0.88	0.53

[a] The binding affinity of KDR variants to $VEGF_{1-109}$ was measured using a competitive radioimmunoassay and ^{125}I-$VEGF_{1-165}$ (NEN Life Science Products Inc., DuPont) as a tracer except that the affinity for KDR 2–3-IgG was measured using biotinylated VEGF as tracer as described under "Experimental Procedures."

[b] ND, not detectable.

To facilitate preparation of monomeric forms of KDR, a Genenase 1 protease cleavage site (18) was engineered at the junction of the last KDR IgG domain and the CH2 domain (19). The cleaved KDR was shown to be monomeric based on its mobility in nonreducing SDS-PAGE and gel filtration. Both the KDR 1–7 and KDR 1–3 monomers bound all three mAbs and equally well to VEGF (Table I). These results show the first three IgG-like domains are sufficient for binding of VEGF whether in monomeric or dimeric forms.

One VEGF Dimer Binds Two Molecules of the Extracellular Domain of KDR—To determine the stoichiometry of binding of $VEGF_{1-109}$ to the extracellular domain of KDR, we systematically varied the ratio of VEGF to KDR and determined the apparent size of the complexes by gel filtration. The glycosylated monomeric KDR 1–7 migrated as a single peak by gel filtration chromatography with an apparent molecular mass of ~250 kDa (Fig. 3A). By comparison, the dimeric KDR 1–7-IgG migrated as a 600-kDa peak (data not shown). Upon addition of one equivalent of VEGF (dimer) per two equivalents of KDR (monomer), a single complex peak was formed of apparent molecular mass ~400 kDa. A minor shoulder was seen that might represent the slight excess KDR monomer in the mixture. The fact that the 2:1 complex is smaller by gel filtration than expected from the sum of the component molecular masses (520 kDa) may be that VEGF aligns the receptor subunits in a more compact fashion.

Further additions of 2 and 3 equivalents of VEGF did not change the position of the high molecular weight peak, and excess VEGF accumulated as the free dimeric hormone (Fig. 3A). The height of the free VEGF peak was small because VEGF contains no tryptophan residues and therefore has a small molar absorbance at 280 nm. When the ratio of KDR to VEGF exceeded 2:1, free KDR 1–7 accumulated as a shoulder. The hV-1 heterodimeric variant of VEGF forms a 1:1 complex with monomeric KDR. This complex migrated at a position that was intermediate between the free KDR 1–7 and the 2:1

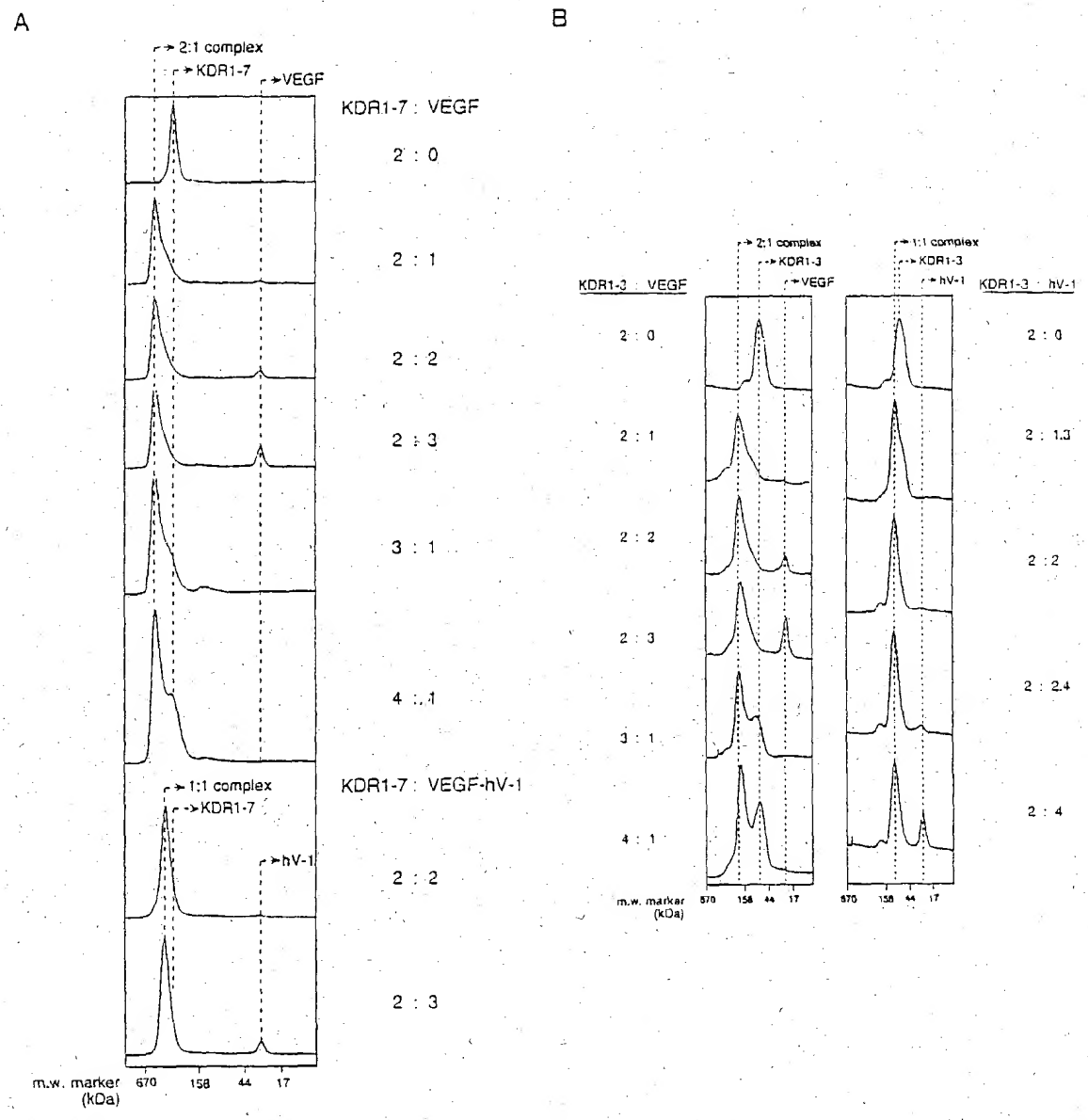

FIG. 3. *Panel A*, gel filtration chromatography of various ratios of KDR 1–7 monomer and VEGF dimer (*upper six chromatograms*) or VEGF heterodimer (hV-1) containing a single functional binding site (*lower two chromatograms*). The concentration of KDR 1–7 monomer was 1 μM except at ratios of 3:1 and 4:1, where the concentration of KDR 1–7 was 1.5 and 2 μM, respectively. The quantitation of protein was determined by amino acids hydrolysis and absorbance at 280 nM. *Panel B*, gel filtration chromatography of various ratios of KDR 1–3 monomer and VEGF dimer (*six chromatograms* on the *left*) or hV-1 (*five chromatograms* on the *right*). The concentration of KDR 1–3 monomer was held constant at 1 μM except at ratios of 3:1 and 4:1, where KDR 1–3 monomer was 1.5 and 2 μM, respectively.

KDR·VEGF complex. When the ratio of the hV-1 to KDR 1–7 exceeded unity, the free heterodimer accumulated in the chromatogram.

Parallel experiments were carried out with the monomeric form of KDR 1–3 (Fig. 3*B*). When no VEGF was present, KDR 1–3 migrated as a single peak of apparent molecular mass of ~70 kDa. Addition of 1 equivalent of VEGF dimer to 2 equivalent of KDR 1–3 resulted in forming a peak with apparent molecular mass of ~160 kDa. Addition of 2 and 3 equivalents of VEGF did not change the position of the complex peak, but free VEGF accumulated. Increasing additions of KDR 1–3 in excess of the 2:1 ratio to VEGF dimer showed increasing appearance of free KDR 1–3. A similar set of experiments with the hV-1 showed it maximally formed a 1:1 complex (Fig. 3*B*); when the ratio of either the variant or KDR 1–3 was skewed from unity, the free excess component accumulated. These experiments explicitly show that two molecules of KDR bind to one VEGF dimer, and that form of KDR lacking IgG-like domains 4–7 are capable of producing the 2:1 complex in solution. When VEGF is engineered to have only one functional binding site (hV-1) it cannot dimerize the receptor *in vitro*.

VEGF Binds Avidly to Dimeric versus Monomeric Forms of KDR—Given the fact that the VEGF dimer binds two molecules of receptor we wished to determine to what extent predimerization of the receptor influenced affinity. This can be readily seen by comparing the binding constants for the monomeric and dimeric forms of KDR (Table I). KDR-IgG fusions containing domains 1–7 or 1–3 bound 50–100-fold stronger

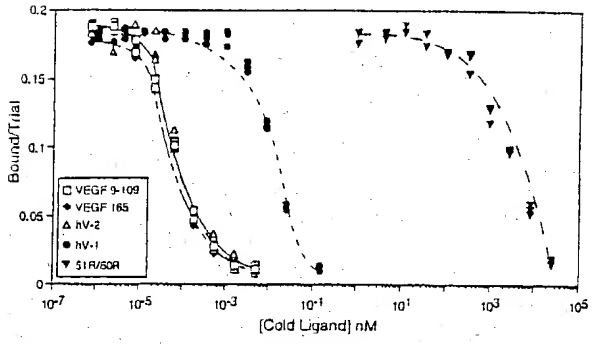

FIG. 4. **VEGF containing two sites binds much stronger to predimerized forms of KDR.** Displacement of ^{125}I-VEGF$_{1-165}$ from the dimeric KDR 1–7-IgG is shown for wild-type VEGF$_{1-165}$, VEGF$_{8-109}$, the single disulfide heterodimer containing both binding sites (hV-2) or single binding site (hV-1), and monomeric VEGF$_{1-109}$, C51R/C60R. The IC_{50} values from here and Fig. 2*B* are summarized in Table II.

than their monomeric counterparts. The affinity of the single-site heterodimeric VEGF for binding to the dimeric KDR IgG fusion was about 200-fold weaker than wild-type VEGF (Fig. 4). In contrast, binding to monomeric KDR for the heterodimer was only 2-fold weaker than native VEGF (Fig. 2*B*). These data, summarized in Table II, show that binding of dimeric VEGF to predimerized KDR is ~100-fold stronger than when either the hormone or receptor contains a single binding site.

VEGF Binds Virtually the Same Way to Monomeric and Dimeric KDR—Given the strong avidity component to binding of VEGF to its receptor, we wished to determine if VEGF binds the same way to monomeric and dimeric forms of KDR 1–7. We have previously reported the alanine scan of VEGF for binding to KDR-IgG (9). Here we analyze the binding of these same alanine mutants to monomeric KDR (Table III). The data show that the same set of alanine mutants that are most disruptive for binding to KDR-IgG are also strongly disruptive to binding monomeric KDR.

There are some subtle and systematic differences in the way the alanine mutants bind the monomeric *versus* dimeric KDR. For example M18A, I43A, I46A, E64A, and I83A were more disruptive to affinity (by factors ranging from 2–8-fold) when tested against the monomeric *versus* dimeric KDR. Only F17A was more disruptive to the dimer than the monomer (by ~2-fold). The biased suppression of the disruptive effects of the alanine mutations when binding to the dimer is likely caused from avidity in binding. We conclude there are no gross differences in the way monomeric and dimeric forms of KDR bind to VEGF.

KDR Domains 1–3 Are Sufficient for Signaling in Cells—NIH 3T3 fibroblast cells that contain the extracellular domain of colony-stimulating factor receptor fused to the transmembrane and intracellular domain of the Flt-4 receptor incorporate [^{3}H]thymidine and proliferate when treated with the colony-stimulating factor (20). To produce a VEGF responsive cell line, we made a similar chimera in which the seven IgG-like domains of KDR were linked to the transmembrane and intracellular kinase domain of Flt-4. At low concentrations of VEGF this cell line incorporated [^{3}H]thymidine with an EC_{50} of ~100 pM (Fig. 5*A*); high concentrations of VEGF (>1 μM) showed inhibition. Such a bell-shaped dose-response curve is anticipated for a two-site hormone dimerizing two identical receptors (21). The hV-1 was inactive (Fig. 5*A*).

A similar construct was produced in which only domains 1–3 of KDR were linked to Flt-4. These cells also incorporated [^{3}H]thymidine in response to VEGF (Fig. 5*B*) but did so with a higher EC_{50} (~10 nM) and lower maximal response. We did not go to high enough concentrations to see inhibition by VEGF. The hV-1 was virtually inactive. Primary human umbilical vein endothelial cells (HuVEC) showed a bell-shaped dose-response curve (Fig. 5*C*). We resist making quantitative comparisons between HuVEC and KDR expressed NIH 3T3 cells given the fact that the HuVEC contain both KDR and Flt-1 receptors (22).

The difference in EC_{50} values and maximal response for the KDR 1–7 and KDR 1–3 cell lines likely resulted from the fact that the number of functional receptors on the KDR 1–3 cell line was at least 10-fold lower based upon binding of ^{125}I-VEGF (data not shown). To explore the effect of receptor number on signaling directly we isolated three different clones of cells that varied over a range of 12-fold in the amount of the KDR 1–7 that specifically bound ^{125}I-VEGF (Fig. 6*A*). The maximal levels of [^{3}H]thymidine incorporation correlated with the number of receptors expressed on these cells and the EC_{50} values correlated inversely with the number of receptors (Fig. 6*B*). It is interesting that the basal levels of [^{3}H]thymidine incorporation correlated with the receptor number as well, suggesting that receptors can preassociate and signal weakly in the absence of exogenous VEGF. All of the transfectants containing the KDR 1–3 construct expressed much lower levels of receptors which may suggest that domains 4–7 are important for high level expression and display of the receptor.

Antagonism of VEGF Receptors by the Single-site Heterodimer of VEGF—Given the ability of hV-1 to bind but not dimerize and activate KDR (Fig. 5), we studied its ability to antagonize signaling of KDR. Indeed the heterodimer antagonizes [^{3}H]thymidine incorporation in the 3T3 cells transfected with the chimeric KDR (1–7)-Flt-4 receptor and HuVEC with an IC_{50} of ~300 and ~20 nM, respectively (Fig. 7). The fact that the heterodimer is less effective on the 3T3-transfected cells *versus* HuVEC likely reflects the fact that the former expresses much higher levels of receptors (Fig. 6*A*).

TABLE II

Summary of dissociation constants (K_d) for binding of monomeric and dimeric forms of VEGF and KDR 1–7

VEGF$_{1-109}$	KDR	
	Monomer	Dimer
Monomer[a]	~20 μM	1.5 μM
Single-site heterodimer (hV-1)[a]	10 nM	7 nM
Wild-type dimer[b]	5 nM	50 pM

[a] Data from Figs. 2*B* and 4. Data for the monomeric VEGF binding to monomeric KDR was from other experiments not shown.
[b] Data from Table I.

TABLE III

The comparison of the effect alanine mutants of VEGF$_{1-109}$ on binding to KDR 1–7-IgG or KDR1–7 monomer

The relative binding affinity was expressed as the fold difference of alanine mutants with VEGF$_{1-109}$ in the competitive binding assay as described under "Experimental Procedures." Standard deviations in these measurements averaged ±25% of the value shown. Residues are shown in two groups (*e.g.* F17A or I43A′) to indicate that they are presented in the same epitope but from different subunits. Each mutant is present twice in the dimer.

	KDR-IgG dimer	KDR monomer
VEGF wt	1	1
F17A	230	91
M18A	11	98
E64A	120	460
I43A′	25	120
Y45A′	140	170
I46A′	400	1000
Q79A′	100	
I83A′	360	810

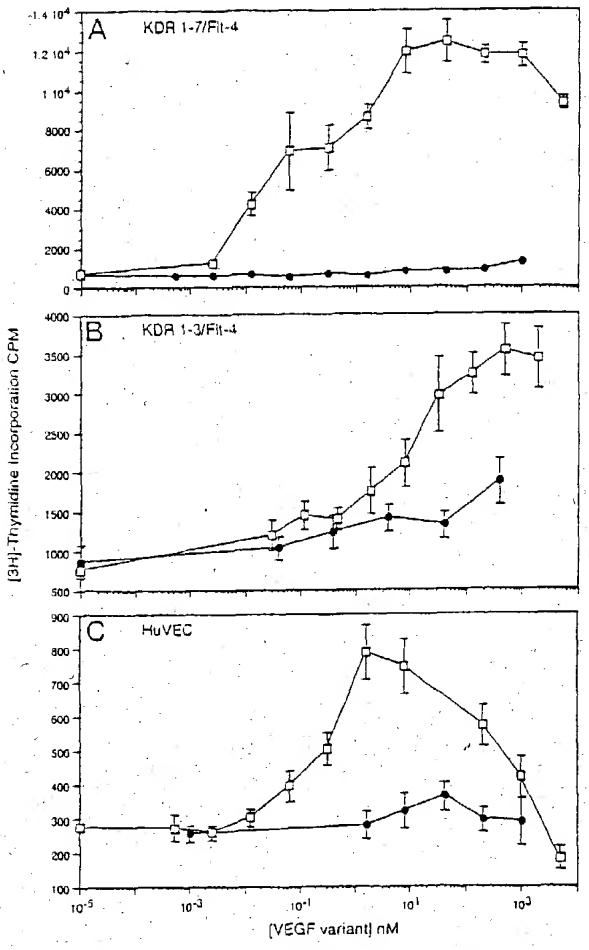

FIG. 5. **The agonistic effects of VEGF variants on DNA synthesis of 3T3 cells stably expressing KDR 1–7 (ECD)/Flt4 (ICD) (*Panel A*), KDR 1–3 (ECD)/Flt-4 (ICD) (*Panel B*), or HuVEC (*Panel C*).** Cells were treated for 18 h with either $VEGF_{9-109}$ (*open squares*) or the $VEGF_{1-109}$ heterodimer with single binding site, hV-1 (*closed circles*). Cells were pulsed with [^{3}H]thymidine for 6 h and analyzed as described under "Experimental Procedures."

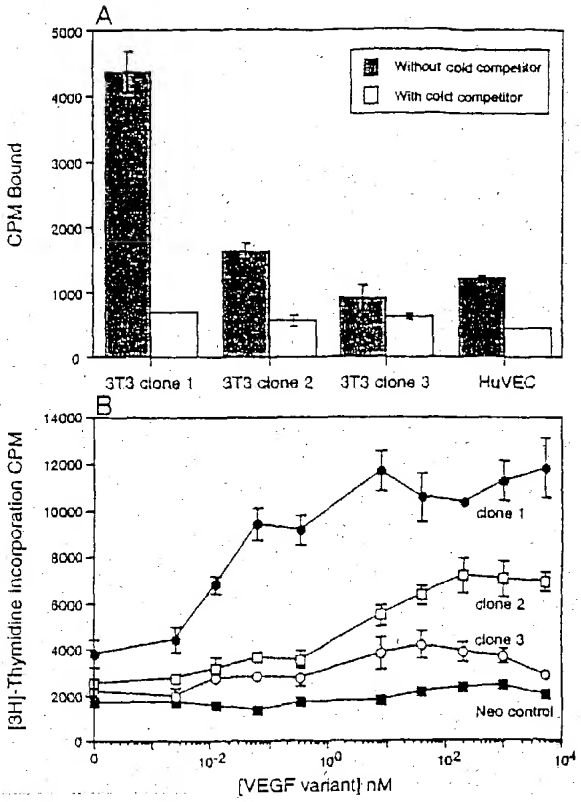

FIG. 6. **3T3 cells stably expressing varying amounts of KDR 1–7 (ECD)/Flt-4 (ICD) receptor respond to $VEGF_{9-109}$ with different values of EC_{50} and maximal response.** *Panel A*, three different 3T3 cell clones expressing varying amounts of the KDR 1–7 (ECD)/Flt-4 (ICD) were isolated and ranked according to the amount of functional binding sites for VEGF as determined by specific binding of ^{125}I-$VEGF_{1-165}$. The same number of 3T3 cells from transfected clones 1, 2, and 3 or HuVEC were plated on the 24-well plate. The ^{125}I-$VEGF_{1-165}$ (0.1 nM) was added with (*open bar*) or without (*filled bar*) a 200-fold molar excess of cold $VEGF_{1-165}$ for 2 h and cells were washed and counted. *Panel B*, these same cell lines were treated with increasing concentrations of $VEGF_{8-109}$ and [^{3}H]thymidine incorporation was measured. The three clones of 3T3 cells expressing KDR 1–7/Flt-4, NEO transfected control cells, and HuVEC were fasted and treated with serial dilutions of $VEGF_{8-109}$ for 18 h. Cells were pulsed with [^{3}H]thymidine for 6 h before harvesting as described under "Experimental Procedures."

DISCUSSION

KDR Binds Across the VEGF Dimer Interface—The data here combined with previous mutational analysis (9) suggest that binding occurs across the VEGF dimer interface (Fig. 2A). It may be a general feature of the cystine-knot hormones that the receptor binds at the interface between hormone subunits. A structure of domain 2 of the Flt-1 receptor bound to VEGF shows it binds across the dimer interface (23). A heterodimer containing one molecule of VEGF linked to its homolog, PLGF, is only 20–50-fold reduced as a mitogen on HuVEC cells (EC_{50} ~ 50 nM) whereas the PLGF homodimers are inactive (24). The fact the VEGF/PLGF heterodimer shows any activity can be rationalized from our mutational analysis (Table III). Some of the critical binding determinants (Phe-17, Glu-64, Gln-79, and Ile-83) are conserved in PLGF and others are reasonably conservative substitutions (M18Q, I43V, Y45H and I46M). These later substitutions would likely have a much more dramatic effect when present in both subunits, thus accounting for the absence of significant mitogenic activity for the PLGF homodimer up to the concentrations that were tested (~1 μM). Similar observations have been made for homodimers and heterodimers of PDGF (isoforms AA, AB, and BB) for binding the PDGF-α and -β receptors (25). Mutational analysis of nerve growth factor, another member of the cystine-knot family of dimeric hormones, shows a broad patch of residues involved in binding receptor that spans the interface between subunits (26).

Requirements and Consequences for Receptor Dimerization—Hormone-induced receptor dimerization is a general mechanism for activation of tyrosine kinase receptors (27). All receptors that bind cystine-knot hormone dimers are presumed to be activated by receptor dimerization (6). Here, gel filtration experiments provide *in vitro* evidence that VEGF binds two molecules of the extracellular domain of KDR. The dimerized complex appears to be very stable since excess VEGF is unable to dissociate the complex to 1:1 complex. This dimerization reaction is critical for signaling because the VEGF heterodimer, hV-1, with only one functional site is inactive in cell-based assays and antagonizes the action of wild-type VEGF. Receptor dimerization is also supported by the observation that cell-based assays show a bell-shaped dose-response curve with respect to VEGF. PDGF isoforms have been shown to induce

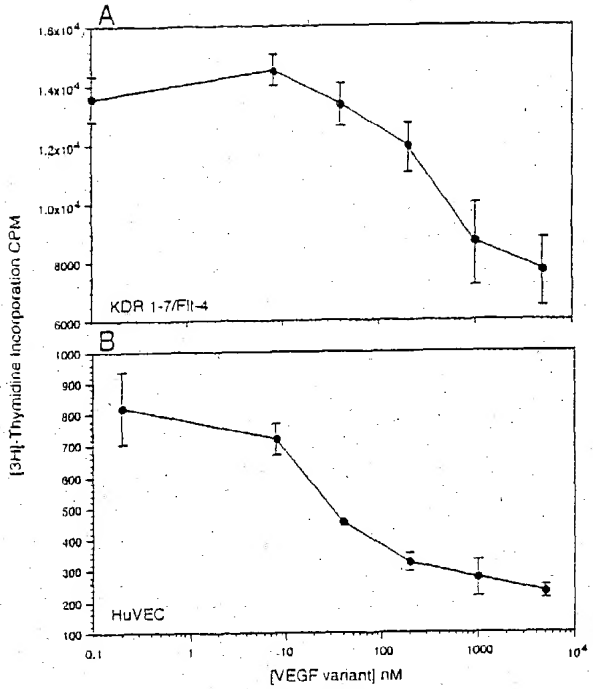

FIG. 7. **The antagonistic effects of VEGF single site heterodimer (hV-1) on DNA synthesis of 3T3 cells stably expressing KDR 1–7 (ECD)/Flt-4 (ICD) (*Panel A*) or HuVEC (*Panel B*).** Cells were incubated with either 0.1 nM $VEGF_{8-109}$ (for 3T3 cells) or 1 nM $VEGF_{8-109}$ (HuVEC) to induce 90% maximal incorporation of [^{3}H]thymidine together with increasing concentrations of the hV-1.

dimerization of the extracellular domains of the PDGF-α and -β receptors *in vitro* (28). Binding of the dimeric hormone, SCF, to the extracellular domain of the Kit receptor, a tyrosine kinase receptor of the IgG class, causes dimerization *in vitro* (29–31), and induces a bell-shaped dose-response curve *in vivo* (30).

Predimerized forms of KDR bind VEGF 100-fold more tightly than monomeric forms of KDR showing a strong avidity component in binding. Dimeric receptor fusion proteins, such as IgG fusions, or receptors bound to monoclonal antibodies are often used as convenient assay reagents for hormones and their variants. The data presented here show that there is a significant avidity component to binding in these fusions that affects the affinity constants. The avidity effect observed here is not the result of an alternate way that VEGF binds the dimeric KDR because alanine mutations in VEGF that are most disruptive to binding monomeric KDR are also the ones that most affect binding to dimeric KDR (Table III).

We observed that wild-type $VEGF_{1-109}$ binds about 100-fold more tightly than the single-site heterodimer to cells expressing KDR 1–7 (not shown). This suggests that receptors on cells may be loosely associated. Moreover, NIH 3T3 cells expressing larger numbers of VEGF receptors showed a higher basal level of [^{3}H]thymidine incorporation in the absence of VEGF (Fig. 6*B*), suggesting that receptors on cells have an intrinsic ability to dimerize in the absence of ligand. Similar observations have been made for cells overexpressing various tyrosine kinase receptors, such as variants of the EGF receptor (27). Overexpression of the PDGF receptors can induce receptor autophosphorylation in the absence of ligand, and it is even possible to cross-link small amounts of the extracellular domains in the absence of PDGF (28). The fact we did not see evidence for dimerization by gel filtration of the ecodomains of KDR 1–7 or 1–3 in the absence of VEGF may only reflect the sensitivity of the method and that the receptors have a much higher effective concentration on cells than in our solution experiments (~1 μM).

Deletion experiments showed that domains 2–3 of KDR are sufficient and necessary for high affinity binding of VEGF (Table I). Cells can signal when transfected with KDR domain 1–3 linked to Flt-4, even with low receptor expression, suggesting that domains 4–7 are not essential for signaling. We cannot rule out other roles for these domains; they may stabilize the signal transduction complex and/or provide for better display and expression of the receptor. Systematic deletion experiments have been conducted on at least four other tyrosine kinase receptors of the IgG class, and generally show that binding is dominated by IgG-like domains 2–3. Deletion experiments showed the first three of the five IgG-like domains in the Kit receptor are required for binding of SCF, but there is uncertainty regarding the role of domain 4 in signaling (30, 31). An antibody directed toward domain 4 blocked signaling in cells transfected with Kit, and deletion of domain 4 blocked signaling but not stem cell factor binding (30). In contrast, biophysical experiments (31) showed that Kit 1–3 can dimerize in solution with stem cell factor and both the enthalpy and free energy of binding were indistinguishable from Kit 1–5. In either case, both groups agree that the ligand-binding site for stem cell factor is contained in the first three IgG-like domains.

Deletion analysis of PDGF-α receptor, which contains five IgG-like domains, has shown that domains 2–3 are sufficient for binding PDGF isoforms although the presence of domain 1 has a small differential effect on binding PDGF-AA *versus* PDGF-BB (32). Deletion analysis of the fibroblast growth factor receptor, which contains three IgG-like domains, showed that domains 2–3 are sufficient for high affinity binding of fibroblast growth factor (33). Deletion experiments in Flt-1, which like KDR contains seven IgG-like domains, have shown that the VEGF-binding site is located among the first three IgG-like domains (34–36) and domain 2 of Flt-1 alone can bind VEGF tightly (23). Thus, domain 2 may plays a dominant role in all five of these tyrosine kinase receptors that have IgG-like domains and may be general to the other members of this class.

Mechanism-based Antagonsits of VEGF—Antagonists to VEGF may be very useful in preventing tumor angiogenesis and retinopathy diseases. Here, we have elucidated the functional requirements for receptor binding and activation and designed an antagonist, hV-1, for the proliferation of HuVEC cells based on this knowledge. The fact the IC_{50} of hV-1 for inhibiting VEGF in HuVEC (~20 nM) is ~100-fold higher than the EC_{50} of VEGF stimulating growth (~0.2 nM) likely reflects the avidity effect described above. We believe the hV-1 antagonize VEGF stimulation of HuVEC by blocking the dimerization of KDR since KDR is more important for signaling mitogensis. However, the hV-1 does bind Flt-1 with near wild-type affinity and we are currently looking at its ability to activate Flt-1. Alanine scanning of both receptor-binding sites on VEGF suggests that the binding sites for KDR and Flt-1 overlap and are not identical (9, 23, 37). Based on these results it should also be possible to design receptor specific antagonists and further elucidate the functions of the two receptors. Overall, these studies provide a basis from which we can produce new analogs of VEGF to both probe its biology and generate new and potent therapeutics.

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REFERENCES

1. Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thieme, H., Iwamoto, M. A., and Park, J. E. (1994) *N. Engl. J. Med.* 331, 1480–1487
2. Folkman, J. (1995) *Nat. Med.* 1, 27–31
3. Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. (1992) *Endocr. Rev.* 13, 18–32
4. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X.-F., Breitman, M. L., and Schuh, A. C. (1995) *Nature* 376, 62–66
5. Fong, G.-H., Rossant, J., Gertsenstein, M., and Breitman, M. L. (1995) *Nature* 376, 66–74
6. Sun, P. D., and Davies, D. R. (1995) *Annu. Rev. Biophys. Biomol. Struct.* 24, 269–291
7. Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V., and Ferrara, N. (1989) *Science* 246, 1306–1309
8. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) *Mol. Endocrinol.* 5, 1806–1814
9. Muller, Y. A., Li, B., Christinger, H. W., Wells, J. A., Cunningham, B. C., and De Vos, A. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 7192–7197
10. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. (1994) *J. Biol. Chem.* 269, 25646–25654
11. Kunkle, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 488–492
12. Chamow, S. M., and Ashkenazi, A. (1996) *Trends Biotechnol.* 14, 52–60
13. Lucas, B. K., Giere, L. M., DeMarco, R. A., Shen, A., Chisholm, V., and Crowley, C. W. (1996) *Nucleic Acids Res.* 24, 1774–1779
14. Potgens, A. J., Lubsen, N. H., van Altena, M. C., Vermeulen, R., Bakker, A., Schoenmakers, J. G. G., Ruiter, D. J., and de Waal, R. M. W. (1994) *J. Biol. Chem.* 269, 32879–32885
15. Graham, F. L., and Van der Eb, A. J. (1973) *Virology* 52, 456–467
16. Williams, A. F., and Barclay, A. N. (1988) *Annu. Rev. Immunol.* 6, 381–405
17. Finnerty, H., Kelleher, K., Morris, G. E., Bean, K., Merberg, D. M., Kriz, R., Morris, J. C., Sookdeo, H., Turner, K. J., and Wood, C. R. (1993) *Oncogene* 8, 2293–2298
18. Carter, P., and Wells, J. A. (1987) *Science* 24, 394–399
19. Beck, J. T., Marsters, S. A., Harris, R. J., Carter, P., Ashkenazi, A., and Chamow, S. M. (1994) *Mol. Immunol.* 31, 1335–1344
20. Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L., and Alitalo, K. (1994) *Oncogene* 9, 3545–3555
21. Fuh, G. Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V., and Wells, J. A. (1992) *Science* 256, 1677–1680
22. Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. (1996) *J. Biol. Chem.* 271, 5761–5767
23. Weismann, C., Fuh, G., Christinger, H. W., Eigenbrot, C., Wells, J. A., and De Vos, A. M. (1997) *Cell* 91, 695–704
24. Cao, Y., Chen, H., Zhou, L., Chiang, M.-K., Anand-Apte, B. Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G., and Tsang, M. L.-S. (1996) *J. Biol. Chem.* 271, 3154–3162
25. Heldin, C. H. (1995) *Cell* 80, 213–223
26. Bradshaw, R. A., Murray-Rust, J., Ibanez, C. F., McDonald, N. Q., Lapatto, R., and Blundell, T. L. (1994) *Protein Sci.* 3, 901–913
27. Ullrich, A., and Schlessinger, J. (1990) *Cell* 61, 203–212
28. Herren, B., Rooney, B., Weyer, K. A., Iberg, N., Schmid, G., and Pech, M. (1993) *J. Biol. Chem.* 268, 15088–15095
29. Lev, S., Yarden, Y., and Givol, D. (1992) *J. Biol. Chem.* 267, 15970–15977
30. Blechman, J. M., Lev, S., Barg, J., Eisenstein, M., Vaks, B., Vogel, Z., Givol, D., and Yarden, Y. (1995) *Cell* 80, 103–113
31. Lemmon, M. A., Pinchasi, D., Zhou, M., Lax, I., and Schlessinger, J. (1997) *J. Biol. Chem.* 272, 6311–6317
32. Mahadevan, D., Yu, J.-C., Saldanha, J. W., Thanki, N., McPhie, P., Uren, A., LaRochelle, W. J., and Heidaran, M. A. (1995) *J. Biol. Chem.* 270, 27595–27600
33. Wang, F., Kan, M., Xu, J., Yan, G., and McKeehan, W. L. (1995) *J. Biol. Chem.* 270, 10222–10230
34. Davis-Smyth, T., Chen, H., Park, J., Presta, L. G., and Ferrara, N. (1996) *EMBO J.* 15, 4919–4927
35. Cunningham, S. A., Stephan, C. C., Arrate, M. P., Ayer, K. G., and Brock, T. A. (1997) *Biochem. Biophys. Res. Commun.* 231, 596–599
36. Barleon, B., Totzke, F., Herzog, C., Blanke, S., Kremmer, E., Siemeister, G., Marme, D., and Martiny-Baron, G. (1997) *J. Biol. Chem.* 272, 10382–10388
37. Keyt, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H., and Ferrara N. (1996) *J. Biol. Chem.* 271, 5638–5646

AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

THIS IS Exhibit PAWR-9

referred to in the Statutory Declaration

of Peter Adrian Walton Rogers

made before me

DATED this 12th Day of November, 2001

(Signature of Witness)

Medical Practitioner